The E2F Transcription Factor Is a Cellular Target for the RB Protein

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Summary

Although it is generally believed that the product of the retinoblastoma susceptibility gene (RB1) is an important regulator of cell proliferation, the biochemical mechanism for its action is unclear. We now show that the RB protein is found in a complex with the E2F transcription factor and that only the underphosphorylated form of RB is in the E2F complex. Moreover, the adenovirus E1A protein can dissociate the E2F–RB complex, dependent on E1A sequence also critical for E1A to bind to RB. These sequences are also critical for E1A to immortalize primary cell cultures and to transform in conjunction with other oncogenes. Taken together, these results suggest that the interaction of RB with E2F is an important event in the control of cellular proliferation and that the dissociation of the complex is part of the mechanism by which E1A inactivates RB function.

Introduction

Inactivation of the retinoblastoma susceptibility gene (RB1) is correlated with the development of a subset of human neoplasias (Friend et al., 1987; Lee et al., 1987; Fung et al., 1987; Horowitz et al., 1990; Yokota et al., 1988; Harbour et al., 1988; Lee et al., 1988; T'Ang et al., 1988; Varley et al., 1989; Weichselbaum et al., 1988). The product of the retinoblastoma gene is a 110 kd nuclear phosphoprotein termed p110-RB (Horowitz et al., 1990; Varley et al., 1989; Lee et al., 1987; Xu et al., 1989). The RB protein is expressed at equivalent levels in all human and mouse cells examined with the exception of tumor cells in which the RB gene has been inactivated by mutation or deletion (Horowitz et al., 1990; Varley et al., 1989; Lee et al., 1987; Xu et al., 1989). Given that it is the loss of RB function that is correlated with the etiology of human tumors, it is widely believed that the RB protein functions to limit or constrain cell proliferation.

The extent of RB phosphorylation varies with the progression of cells through the cell cycle (DeCaprio et al., 1989; Buchkovitch et al., 1989; Chen et al., 1989; Mihara et al., 1989). Quiescent cells or cells in early G1 contain largely un- or underphosphorylated p110-RB. As cells progress toward S phase, RB becomes phosphorylated and remains so until dephosphorylation occurs in late

stages of mitosis. The amino acid sequence of p110-RB contains consensus sites for phosphorylation by cdc2, a kinase whose activity is regulated during the cell cycle, and there is both direct and indirect evidence that supports the notion that RB is a target for cdc2 phosphorylation (Cooper and Whyte, 1989; Taya et al., 1989; Shenoy et al., 1989).

The products of several viral oncogenes, including adenovirus E1A, SV40 and polyomavirus large T antigen, and human papillomavirus (HPV) E7, interact with the RB protein, dependent on sequences that are essential for transforming function, leading to the speculation that this interaction inactivates the normal function of RB (Whyte et al., 1988, 1989; DeCaprio et al., 1988; Dyson et al., 1989; Münger et al., 1989; Egan et al., 1989). Consistent with the hypothesis that the active (i.e., growth suppressing) forms of RB are un- or underphosphorylated, SV40 large T antigen specifically targets these molecules for binding and presumably functional inactivation (Ludlow et al., 1989). It thus appears likely that p110-RB functions to restrict the G1/S transition; it is then the phosphorylation of the RB protein or the association with viral oncogene products that inactivates this function and allows the cell to proceed through the cell cycle. Recently, a variety of uncharacterized cellular proteins have been shown to associate with RB in vitro (Kaelin et al., 1991; Huang et al., 1991). The binding of these cellular proteins was shown to be dependent on sequences known to be required for the association of RB with viral oncoproteins. Clearly, an elucidation of the normal role of the RB protein in controlling cell proliferation, including the identification of the cellular targets of its action, is critically important.

Given the nuclear location of the RB protein and the apparent affinity of the protein for DNA (Lee et al., 1987; Horowitz et al., 1989), a role for RB in transcription control has been suggested. Indeed, transfection assays indicate that the RB protein can repress transcription of the c-fos gene, dependent on a 30 bp sequence element of the c-fos promoter (Robbins et al., 1990). Moreover, indirect evidence suggests that RB may also repress the c-myc promoter, since the TGF-β-induced repression of c-myc transcription can be abrogated by viral oncoproteins that are competent to interact with the RB protein (Pietenpol et al., 1990). Very recent experiments have suggested that in certain cell types RB may stimulate, rather than repress, RB control element (RCE)-dependent transcription, particularly from the TGF-β1 promoter (Kim et al., 1991). Taken together, these results suggest that RB may function to repress immediate-early gene transcription, either directly (fos) or indirectly (TGF-β1), thus limiting the progression of cells through the cell cycle.

We have recently shown that the adenovirus E1A protein can dissociate complexes containing the E2F transcription factor, releasing free E2F (Bagchi et al., 1990). The ability of E1A to dissociate the E2F complexes is dependent on sequences that are also important for oncogenic activity of E1A (Raychaudhuri et al., 1990). These are also se-

quences that are shared with SV40 large T antigen and HPV E7 and that are involved in the interaction with cellular proteins such as RB. Since the process of E1A-mediated dissociation of the E2F complexes could certainly involve a direct interaction of E1A with the complex, we have investigated the possibility that the RB protein is part of one such E2F complex.

Results

An E2F-Containing Complex Is Recognized by an RB Antibody

The cellular transcription factor E2F, initially identified as a component of the adenovirus E2 promoter transcription complex and a target for transactivation by E1A (Kovesdi et al., 1986; Yee et al., 1989), is found in all cell types thus far assayed. Although initial experiments utilizing HeLa cell extracts suggested that E2F bound to DNA on its own, recent experiments have shown that E2F is complexed with other cellular factors in most cell types (Bagchi et al., 1990). An example of these interactions involving E2F is seen in the analysis shown in Figure 1. An extract of the human promonocytic cell line U937 was assayed for E2F-binding activity using a probe derived from the adenovirus E2 promoter. Two major DNA–protein complexes, labeled E2F$_c$ and E2F$_c$·, are detected by this assay (Figure 1, left). An additional, minor complex that migrates between the two major species is not reproducibly observed. If the extract is first treated with deoxycholate and then assayed for E2F binding, the initial complexes are converted into a single DNA–protein complex that is typical of the interaction of a free E2F factor with the DNA, based on our previous observations (Bagchi et al., 1990). Competition with a DNA probe containing the wild-type E2F recognition sequence but not a mutant E2F recognition sequence demonstrates the specificity of the interactions (Figure 1, right).

Previous experiments have shown that the adenovirus E1A protein can dissociate E2F-containing complexes, releasing free E2F (Bagchi et al., 1990). Although the precise mechanism for this dissociation has not been established, one possibility could involve a direct interaction of E1A with the complex, displacing E2F and leaving E1A associated with the protein originally bound to E2F. There are, of course, a number of candidates for the protein that would be left in a complex with E1A; these are the proteins that are coimmunoprecipitated with E1A in extracts of E1A-expressing cells (Yee and Branton, 1985; Harlow et al., 1986). Indeed, one such protein, cyclin A, is involved in an interaction with E2F that is dissociated by the E1A protein (Mudryj et al., 1991). Of course, the product of RB1 is another E1A-associated protein that is a potential candidate for an E2F-binding protein (Whyte et al., 1988).

Using antibodies that recognize the RB protein, we have tested the U937 extracts for the possible involvement of RB in the formation of the E2F-specific complexes. The addition of an RB monoclonal antibody to the extract did not alter the slowly migrating E2F$_c$ complex. In sharp contrast, the faster-migrating E2F$_c$· complex was completely eliminated by the addition of the RB-specific monoclonal antibody, thus indicating that this complex did indeed con-
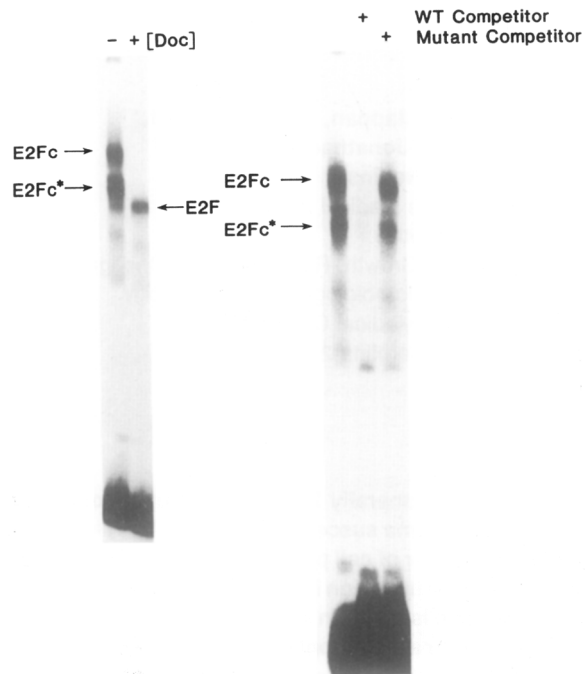

Figure 1. Extracts of U937 Cells Contain Multiple E2F Complexes

(Left) Dissociation by deoxycholate. E2F-binding assays utilized a whole-cell extract (15 μg) prepared from U937 cells and a DNA probe (0.5 ng) that derives from the adenovirus E2 promoter. Conditions for DNA binding and gel electrophoresis are described in Experimental Procedures. One-half of the sample was treated with deoxycholate followed by NP-40 prior to the assay for DNA binding as described in Experimental Procedures.

(Right) Sequence specificity of DNA binding. E2F binding was assayed on the E2 promoter probe in the presence of 200 ng of cold wild-type competitor DNA or 200 ng of a competitor DNA with a mutation in the E2F recognition site that has previously been shown to eliminate E2F binding (Hiebert et al., 1989).

tain the RB protein. The addition of a control antibody (a monoclonal specific to the c-fos protein) to the U937 extract prior to the DNA-binding assay did not alter the pattern of E2F complexes (Figure 2A).

Separate E2F Complexes Contain RB and Cyclin A

Our recent experiments have demonstrated that an S phase–specific E2F complex in NIH 3T3 cells contains the cyclin A protein (Mudryj et al., 1991). The mobility of this 3T3 complex is similar to that of the E2F$_c$ complex detected in the U937 extracts. Indeed, as shown in Figure 2B, the E2F$_c$ complex does involve an interaction with the cyclin A protein. As before, addition of the RB antibody eliminated the faster-migrating E2F$_c$· complex but had no effect on the E2F$_c$ complex. By contrast, addition of the cyclin A antiserum eliminated the E2F$_c$ complex but did not affect the E2F$_c$· complex. It thus appears clear that two distinct E2F complexes can be detected in extracts of growing U937 cells; one involves an interaction with the cyclin A protein and another involves the RB protein. Thus, two of the identified E1A-interacting cellular proteins are found in E2F complexes.

A.

B.
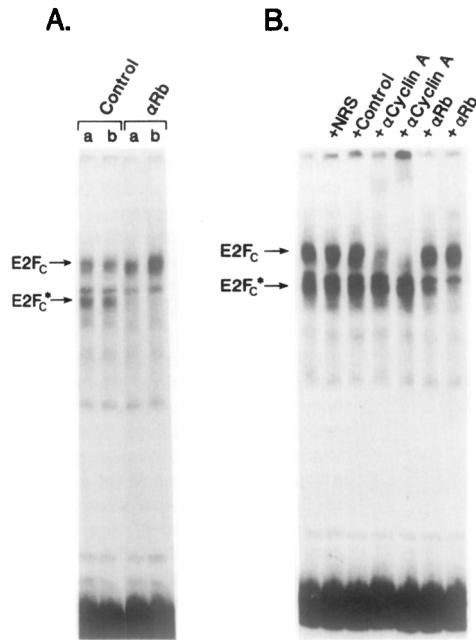
Figure 2. An E2F Complex Contains the RB Protein

(A) The U937 extract was assayed for E2F binding as described in Figure 1. Assays were conducted in the presence of a control monoclonal antibody that was raised against the c-fos protein (c-fos Ab1; Oncogene Science) (Control) and an RB-specific monoclonal antibody (RB-Ab1; Oncogene Science) (αRb). In each case, either a 1:4 (lanes a) or a 1:2 (lanes b) dilution was added.

(B) Separate E2F complexes contain RB and cyclin A. The U937 extract was assayed for E2F binding as described in Figure 1. Assays were conducted in the presence of normal rabbit serum (NRS) diluted 1:8, the c-fos control monoclonal antibody (Control) diluted 1:2, a rabbit antiserum specific to the cyclin A protein (αCyclin A) diluted 1:16 (left) or 1:8 (right), and the RB-specific monoclonal antibody (αRb) diluted 1:4 (left) or 1:2 (right).

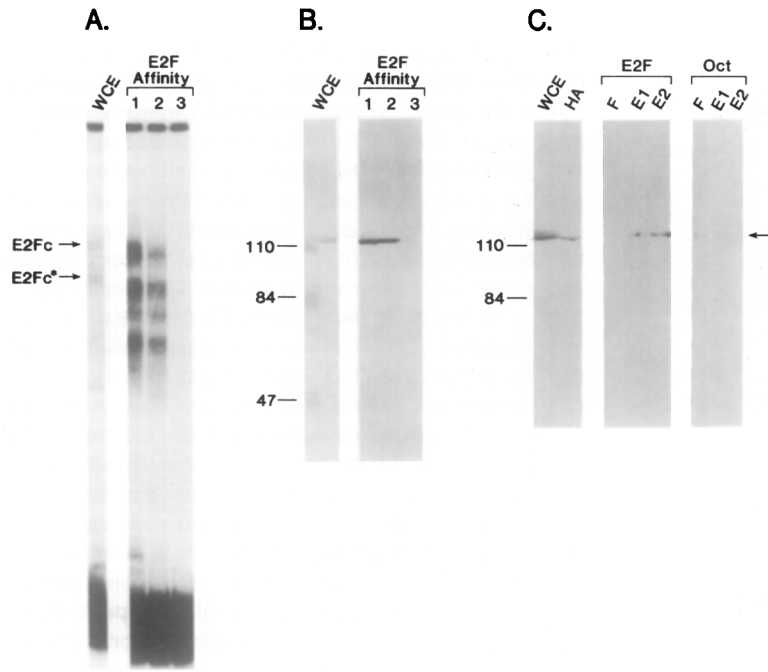## The E2F–RB Complex Is Not Detected in Several RB-Negative Cell Lines

Further evidence that the E2F$_c$· complex was recognized by the RB antibody was provided by an immunoprecipitation analysis. The U937 extract was incubated with an RB monoclonal antibody (RB1), and the immunoprecipitated material was washed and then eluted by the addition of deoxycholate. The released material was then assayed for E2F-binding activity in the presence or absence of an E2F competitor DNA. As seen in Figure 3A, specific E2F-binding activity was clearly detected in the material immunoprecipitated with RB1. We have also utilized several RB-specific antisera that were raised against synthetic peptides derived from the RB protein sequence. As shown in Figure 3A, two of the three peptide sera did precipitate E2F activity (lanes b). Moreover, in each case, preimmune antiserum did not coprecipitate E2F (lanes a), and most importantly, the peptide to which the antiserum was raised was able to block the immunoprecipitation (lanes c). That one of the peptide antibodies (RB140) did not immunoprecipitate the complex suggests that this epitope is either hidden in the complex or has an altered conformation when RB is complexed with E2F.

Finally, we have assayed for the E2F–RB interaction in other cell extracts, including two cell lines deficient in functional RB activity. As shown in Figure 3B, E2F was coprecipitated with the RB monoclonal antibody from extracts of EJ cells and Jurkat cells as well as the U937 cells. However, we could detect no evidence of E2F coprecipitation from extracts of either J82 cells or MGHU-5 cells, both of which lack functional RB protein (Horowitz et al., 1989, 1990). Interestingly, the RB1 gene is expressed in J82 cells but it encodes a mutant form of the RB protein that also fails to interact with E1A (Horowitz et al., 1989).

A.

B.
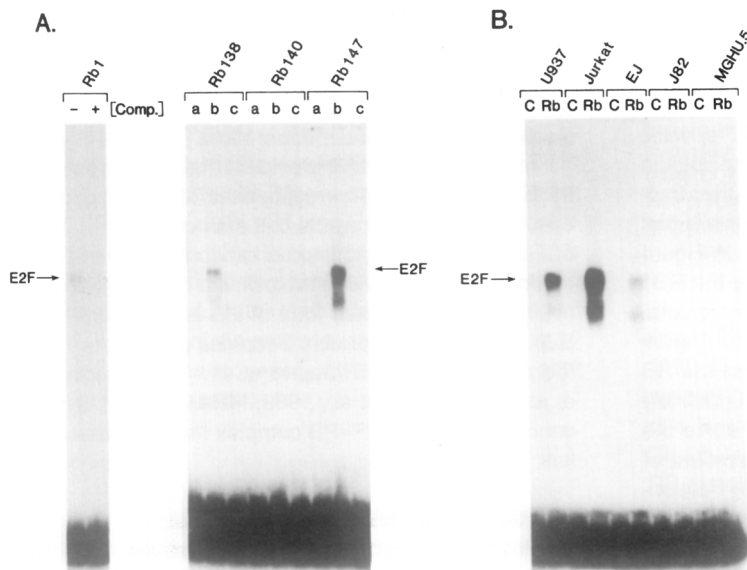
Figure 3. The E2F–RB Complex Is Not Detected in RB-Negative Cells

(A) (Left) A U937 whole-cell extract was immunoprecipitated with the RB-Ab1 monoclonal antibody as described in Experimental Procedures. The supernatant after DOC release was treated with NP-40 and assayed for E2F DNA-binding activity. The specificity of the E2F binding assay was demonstrated by performing the assay in the presence or absence of 200 ng of cold probe DNA. (Right) A U937 whole-cell extract was incubated with 10 μl of preimmune serum (lanes a) or the corresponding antiserum raised against an RB peptide (lanes b). To demonstrate the specificity of the coprecipitation, the RB antisera were preincubated with 10 μg of the corresponding peptide prior to the immunoprecipitation (lanes c). Conditions for the immunoprecipitation were the same as used for the monoclonal antibody.

(B) Immunoprecipitation of extracts of RB+ and RB− cells. Immunoprecipitations were carried out as described in (A) using the c-fos monoclonal antibody as a control (lanes C) or the RB-Ab1 monoclonal antibody (lanes Rb). Whole-cell extracts were prepared from the indicated cell lines, and 100 μg of each was employed for the assay.

A.

E2Fc →
E2Fc° →

B.

110—
84—
47—

C.

110—
84—

Figure 4. The RB Protein Copurifies with E2F

(A) A U937 whole-cell extract was chromatographed on a heparin–agarose column as described previously in Experimental Procedures. The heparin–agarose eluate was incubated with 2 mg of salmon sperm DNA to block nonspecific DNA binding. This preparation was chromatographed on an E2F DNA affinity column as described in Experimental Procedures. The column was eluted with buffer containing 600 mM KCl, and 1 ml fractions were collected. E2F-binding activity was assayed (as described in Figure 1) in 1 µl of the whole-cell extract and 3 µl of the affinity eluate fractions. (B) Western blot assay of samples from affinity purification. The affinity column eluate fractions were concentrated by TCA precipitation, redissolved in SDS sample buffer, and applied to an 8% SDS–polyacrylamide gel. In addition, a 30 µl aliquot of the whole-cell extract was also applied to the gel. After electrophoresis, the separated polypeptides were electrophoretically transferred to nitrocellulose. The blot was incubated with an RB peptide antiserum (RB-Ab2; Oncogene Science) at 10 µg/ml followed by incubation with horseradish peroxidase–conjugated protein A (ABC kit; Vector Laboratories). Stained marker proteins are in the left lane.

(C) RB is specifically retained on the E2F column. A U937 whole-cell extract was processed as described in (B) and applied in parallel to an E2F affinity column and an octamer-specific affinity column. The two columns were eluted with 600 mM KCl, and 1 ml fractions were collected. The eluate fractions were TCA precipitated and dissolved in SDS sample buffer, and the entire samples were analyzed. Due to the high concentration of protein in the affinity column loading material (HA) as well as the affinity flowthrough fractions (F), only 10% of the volume of these fractions was applied to the gel. Finally, a 30 µl aliquot of the whole-cell extract (WCE) was also analyzed.

The RB Protein Copurifies with E2F

The experiments presented in Figures 2 and 3 strongly suggest that the RB protein is a component of the E2F$_c$· complex. As an additional proof of this interaction, we have reversed the procedure; that is, we have isolated the E2F factor and assayed for the presence of the RB protein. Although an E2F-specific antiserum is not available, it is possible to isolate E2F by DNA affinity chromatography and then assay for the presence of a 110 kd polypeptide that is immunoreactive with the RB monoclonal antibody. A whole-cell extract of U937 cells was fractionated through a heparin–agarose column followed by an E2F-specific DNA affinity column. Fractions from the affinity column eluate were assayed for E2F-binding activity and then fractionated on an SDS–polyacrylamide gel for Western blot analysis with the RB antibody. As can be seen in Figure 4B, the 110 kd RB polypeptide, as detected by the RB1 antibody, is indeed detected in the affinity column eluate, coinciding with the presence of the E2F$_c$· complex (Figure 4A). It should also be noted that the recovery of the RB polypeptide in the E2F affinity column eluate is not likely the result of simple nonspecific DNA binding since the sample is loaded in the presence of a large amount of salmon sperm DNA. This was in fact confirmed by fractionating a U937 extract on two parallel DNA affinity columns, one containing the E2F-binding site and the other containing the octamer element–binding site. As shown in Figure 4C, the RB protein is again recovered in the E2F affinity eluate but is not recovered in the eluate of the octamer column.

A variety of previous experiments have shown that in extracts of asynchronously growing cells there is a mixture of phosphorylated and unphosphorylated forms of the RB protein that can be resolved by SDS–PAGE since the phosphorylated forms of the protein migrate more slowly than the unphosphorylated protein (DeCaprio et al., 1989; Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989). It is apparent from the analysis of Figure 4B that the crude extract (WCE) contains several species that react with the RB antibody, whereas the affinity column eluate appears to contain a single polypeptide. This is more clearly shown in an independent analysis, with improved gel resolution, in which it is evident that the polypeptide in the E2F affinity eluate corresponds to the fastest-migrating species of RB in the whole-cell extract (Figure 5). Thus, E2F does not appear to interact randomly with the population of RB proteins within the cell, but rather, only the un- or underphosphorylated form of RB is in a complex with E2F. Since the RB protein becomes phosphorylated as the cell leaves G1 (DeCaprio et al., 1989; Buchkovitch et al., 1989; Chen et al., 1989; Mihara et al., 1989), we conclude that the E2F–RB complex must be most prevalent in G1.

E1A Dissociates the E2F–RB Complex

Previous experiments have shown that the adenovirus E1A protein can dissociate E2F-containing complexes, releasing free E2F, and that this activity correlates with the ability of the 12S E1A product to transactivate transcription (Bagchi et al., 1990). Moreover, the E1A sequences re-
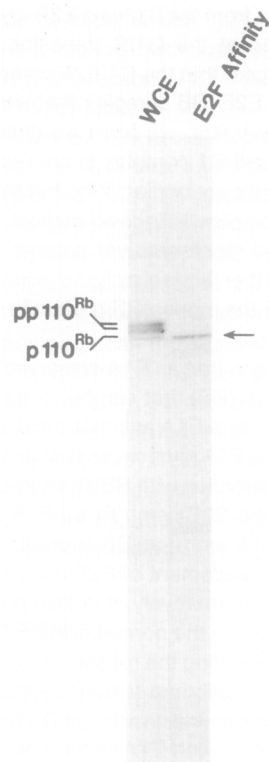
Figure 5. The Underphosphorylated Form of RB Is in the E2F Complex

Samples of a U937 whole-cell extract and the E2F DNA affinity column eluate of this extract were assayed as described in Figure 4B.
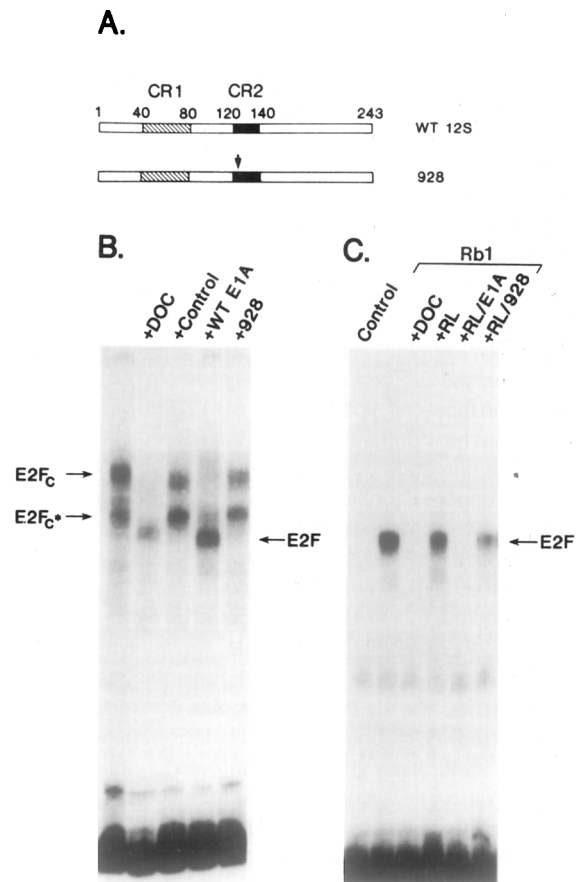

Figure 6. The Adenovirus E1A Protein Can Dissociate the E2F–RB Complex

(A) Schematic depiction of the structure of the wild-type E1A 12S cDNA and the 928 mutant cDNA.

(B) Assays for E1A dissociation of the E2F–RB complex. The U937 whole-cell extract (15 μg) was preincubated with a control reticulocyte lysate or equal amounts (1 μl) of a reticulocyte lysate programmed with transcripts of the wild-type E1A 12S cDNA or the 928 mutant. After incubation for 15 min, the extract was assayed for E2F DNA-binding activity by gel retardation. A parallel sample of U937 extract was treated with deoxycholate and assayed for E2F.

(C) Immunoprecipitation assays. A U937 whole-cell extract was immunoprecipitated with a control monoclonal or the RB monoclonal antibody as described in Experimental Procedures. Prior to immunoprecipitation with the RB antibody, the extract was incubated for 30 min with 0.8% DOC, or 12 μl of control reticulocyte lysate, reticulocyte lysate programmed with the 12S E1A RNA, or reticulocyte lysate programmed with the 928 mutant RNA.

quired for this activity correlate with those required for interaction with the RB protein (Bagchi et al., 1990; Raychaudhuri et al., 1991). As shown in Figure 6, the 12S E1A product can dissociate the majority of the two major E2F complexes found in the U937 cell extract, including the E2F complex that contains the RB protein (E2F$_{c'}$). Moreover, the dissociation of the RB-containing E2F complex is dependent on the CR2 sequence of the E1A protein, as indicated by the failure of the 928 point mutant to dissociate the complex. This conclusion is further supported by immunoprecipitation assays in which the ability of E2F to coimmunoprecipitate with RB was abolished by incubation of the U937 extract with wild-type E1A protein but not the 928 mutant (Figure 6C). Since this CR2 sequence is also required for E1A to form a stable complex with the RB protein (Whyte et al., 1989; Stein et al., 1990), we suggest that at least a part of the proposed inactivation of RB function by E1A is a consequence of the ability of E1A to alter the E2F–RB interaction.

Discussion

Inactivation of retinoblastoma gene function is correlated with deregulated control of cell growth and tumorigenesis. In addition, the ability of several viral oncogenes to transform mammalian cells is tightly linked to their ability to bind to the RB protein, leading to the suggestion that this interaction also inactivates RB function (Whyte et al.,

1988, 1989; DeCaprio et al., 1988; Dyson et al., 1989, 1990; Münger et al., 1989). Together, these data strongly support the notion that the RB protein performs a central role in the regulation of mammalian cell proliferation. Clearly, an elucidation of the mechanism of action of the RB protein will be critical to our understanding of the normal control process regulating the transition from quiescence to proliferation as well as how this process can be disrupted during oncogenic transformation. The identification of a cellular target for the RB protein is an important step in the elucidation of the mechanism of action of this growth regulatory gene. The experiments we present here

demonstrate that the E2F transcription factor is one such target.

The E2F transcription factor was originally identified as an E1A-targeted component of the functional transcription complex of the adenovirus E2 promoter (Kovesdi et al., 1986; Yee et al., 1989). A series of experiments has defined the role of E2F in adenovirus E2 transcription as well as the actions of viral regulatory proteins that allow utilization of E2F (Kovesdi et al., 1986; Yee et al., 1989; Loeken and Brady, 1989; Raychaudhuri et al., 1990; Hardy et al., 1989; Huang and Hearing, 1989; Neill et al., 1990). Particularly relevant are recent experiments that have shown that the E2F factor is normally complexed to cellular proteins in most cell types (Bagchi et al., 1990). The results we present here demonstrate that the RB protein is a component of one such complex. Moreover, the finding that only the unphosphorylated or underphosphorylated form of RB is found complexed to E2F suggests that this interaction is most prevalent in the G1 phase of the cell cycle, the time at which RB is believed to exert its action.

Although the E2F–RB complex could be either an active form of E2F or an inactive molecule, we believe the available data suggest that the free E2F molecule is the active form and thus that the E2F in a complex is inactive. Transfection assays demonstrate that an E2F-dependent promoter is inefficiently expressed in cells containing E2F largely in a cyclin A complex, whereas cotransfection of E1A stimulates expression, dependent on E1A sequences that are essential for dissociation of the complexes (Bagchi et al., 1990; Mudryj et al., 1991; Raychaudhuri et al., 1991). A role for RB as a negative regulator of transcription factor activity is also consistent with the recent observations that demonstrate a repression of the c-fos promoter by RB (Robbins et al., 1990). Interestingly, the c-fos promoter element that is essential for repression by RB (the RCE) contains two binding sites for E2F (S. Hiebert, J. M. Horowitz, and J. Nevins, unpublished data), although we do not as yet know whether these E2F sites within the RCE are indeed the critical target for repression by RB. We therefore suggest that the interaction of RB with E2F may block its function as a transcription factor, as appears to be the case for the interaction of cyclin A with E2F (Mudryj et al., 1991). The release of E2F from the RB complex, perhaps as a result of phosphorylation of RB at the G1/S transition, or through the action of E1A in adenovirus-transformed cells, would then allow E2F to activate appropriate target genes. The potential targets, those genes that contain E2F-binding sites, might include genes that are activated prior to DNA synthesis. At least one such gene, that encoding dihydrofolate reductase (DHFR), does possess E2F-binding sites that are of functional importance (Blake and Azizkhan, 1989).

Our recent experiments have shown that the E2F–cyclin A complex is restricted to the S phase of the cell cycle in NIH 3T3 cells (Mudryj et al., 1991). The results we present here suggest, albeit indirectly, that the E2F–RB complex predominates in the G1 phase of the cell cycle, based on the observation that only the underphosphorylated form of RB is bound to E2F. The analysis of E2F interactions in synchronized NIH 3T3 cells also revealed a G1-specific

complex, distinct from the S phase E2F–cyclin A complex, that disappeared at the G1/S transition. Although it is tempting to suggest that the G1 E2F complex detected in 3T3 cells is the E2F–RB complex that we have detected in human cell extracts, we have no direct evidence for such. The 3T3 cell G1 complex is not recognized by the various RB-specific antibodies. Whether this indicates that this 3T3 cell complex is indeed distinct from the E2F–RB complex and represents yet another E2F-containing complex, or whether this is simply the failure of the antibodies to recognize the mouse RB protein in the complex, is not yet clear.

The observations that an E1A-dissociable E2F complex contains a protein (RB) that can be found alternatively in a stable complex with E1A and that the dissociation of the complex requires E1A sequences that are also necessary for the stable interaction with RB strongly suggest that the dissociation of the E2F complex by E1A may involve an interaction of E1A with the RB protein in the complex, resulting in a displacement of E2F and a stable complex of E1A and RB. Alternatively, it is also possible that E1A binds to RB following the normal dissociation of the E2F–RB complex, preventing the reassociation of E2F and RB, and driving the equilibrium toward dissociation. We propose that at least one inactivation of RB function resulting from the interaction with E1A is the loss of the E2F–RB complex. This is further supported by the finding that another one of the E1A-associated proteins, the 60 kd cyclin A protein (Pines and Hunter, 1989), is also found in an E2F-containing complex that can be dissociated by E1A. The fact that two of the E1A-associated proteins are in E2F complexes and that these complexes are targeted by E1A for dissociation suggests that the other E1A-interacting proteins may also be bound to cellular transcription factors in a like manner. If true, then it is also likely that other cellular transcription factors are targeted by these proteins. For instance, the hsp70 promoter and the PCNA promoter are both activated by 12S E1A (Simon et al., 1987; Zerler et al., 1987; Morris and Mathews, 1990), yet neither promoter utilizes the E2F factor.

Finally, we believe these findings provide insight into the normal function of the RB protein and also provide a picture for how the action of the E1A protein could lead to a loss of cellular proliferation control. We have shown that complexes containing the E2F transcription factor are dissociated by the E1A gene product, dependent on E1A sequences that are essential for transformation. We have also shown that there are distinct complexes involving both the RB protein and the cyclin A protein. Moreover, we have shown that the E2F–cyclin A interaction is regulated during the cell proliferation cycle (Mudryj et al., 1991) and that the E2F–RB interaction is restricted to that form of RB that has been suggested to be the active form in G1. It thus appears that the E2F factor may be a critical target for cellular regulatory interactions that control progression through the cell cycle. Previous experiments have established a tight linkage between the ability of E1A to interact with the RB protein, as well as the cyclin A protein, and the ability of E1A to immortalize primary cell cultures and transform in conjunction with the ras oncogene. Based on

these observations, together with the knowledge that the RB protein plays a role in growth suppression, it has been proposed that the interaction of E1A with RB resulted in an inactivation of RB function.

Our results now show that at least a part of this inactivation is the ability of E1A to alter and disrupt the formation of the E2F–RB complex. We also suspect that this function of E1A is likely to be common to the other viral oncogene products that interact with RB, including SV40 large T antigen and HPV E7. In addition to the fact that each of these proteins binds to RB via related protein sequences, it is also true that each can transactivate the adenovirus E2 promoter, dependent on the E2F sites (Loeken and Brady, 1989). More directly, our recent experiments have shown that the E7 protein can indeed disrupt the formation of the E2F–RB complex. We therefore suggest that the disruption of these E2F regulatory events, as a result of the interaction of E1A with the RB protein and the cyclin A protein, may be a contributing factor in the loss of cellular proliferative control.

Experimental Procedures

Cells
U937 and Jurkat cells were maintained as suspension cultures in RPMI 1640 medium containing 10% fetal calf serum. EJ carcinoma cells and the J82 and MGHU-5 retinoblastoma cell lines were maintained as monolayers in DMEM containing 10% fetal calf serum.

Preparation of Extracts
Whole-cell extracts were prepared as previously described (Bagchi et al., 1990).

E2F Assays
The assay of E2F-binding activity by gel retardation has been previously described (Yee et al., 1989; Bagchi et al., 1990). Assays contained 15 μg of whole-cell extract and 0.5 ng of ^{32}P-labeled DNA probe, which was a EcoRI–HindIII fragment from the ATF(–) adenovirus E2 promoter plasmid (Loeken and Brady, 1989).

Antibodies
The RB monoclonal antibody Ab1, the RB peptide antiserum Ab2, and the c-fos monoclonal antibody Ab1 were obtained from Oncogene Science. The cyclin A antiserum is a rabbit antiserum (Pines and Hunter, 1989), generously provided by J. Pines and T. Hunter. The RB peptide antisera RB138, RB140, and RB147 have been described previously (Whyte et al., 1988; Bernards et al., 1989).

Immunoprecipitations
Whole-cell extracts (100–150 μg) were incubated with the RB-Ab1 monoclonal antibody or one of the peptide antisera for 1 hr at 4°C in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl$_2$, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT, and 3 mg/ml BSA. The mixtures were then further incubated with protein A–Sepharose for 1 hr at 4°C. The beads were centrifuged and washed four times with 800 μl of the same buffer and then treated with 6 μl of the buffer containing 0.8% deoxycholate. The beads were centrifuged, and the supernatant was treated with NP-40 and used for an E2F DNA-binding assay.

DNA Affinity Chromatography
A whole-cell extract was prepared from 3 liters of U937 cells (250 mg of protein) and chromatographed on a heparin–agarose column as described previously (Yee et al., 1989). The material eluting at 600 mM KCl was diluted with 4 vol of buffer (20 mM HEPES [pH 7.6], 10% glycerol, 0.1 mM EGTA, 0.1 mM DTT, 0.2 mM PMSF) and incubated with 2 mg of salmon sperm DNA to block nonspecific DNA binding. This preparation was chromatographed on a 1 ml E2F DNA affinity column containing 800 μg of the EcoRI–HindIII fragment from the

ATF(–) adenovirus E2 promoter plasmid (Loeken and Brady, 1989) or a parallel column containing a polymerized octamer element (ATTTG-CAT) derived as a DdeI–HinfI fragment from the immunoglobulin heavy chain enhancer. The column was washed with the same buffer containing 100 mM KCl and then eluted with buffer containing 600 mM KCl. Fractions of 1 ml were collected.

Western Blot Analysis
Aliquots of whole-cell extract or fractions from the affinity purification were concentrated by TCA precipitation, redissolved in SDS sample buffer, and applied to an 8% SDS–polyacrylamide gel. After electrophoresis, the separated polypeptides were electrophoretically transferred to nitrocellulose. The blot was incubated with an RB peptide antiserum (RB-Ab2; Oncogene Science) (10 μg/ml) followed by incubation with horseradish peroxidase–conjugated protein A (ABC kit; Vector Laboratories).

E1A-Mediated Dissociation of E2F Complexes
The procedures for the synthesis of wild-type 12S E1A protein and the 928 mutant protein in reticulocyte lysates have been described (Bagchi et al., 1990).

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